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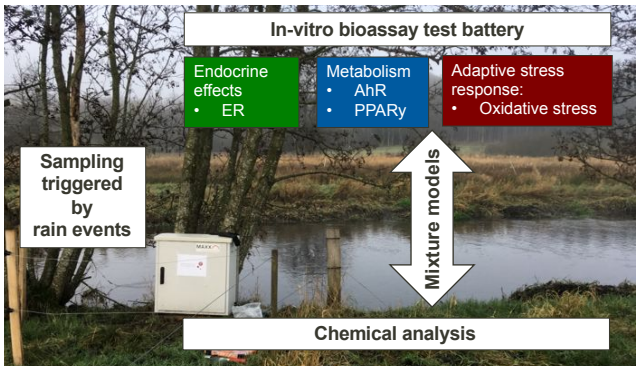

Abstract. Rain events may impact the chemical pollution burden in rivers. Forty-four small streams in Germany were profiled during several rain events for the presence of 395 chemicals and five types of mixture effects in in-vitro bioassays (cytotoxicity, activation of the estrogen, aryl hydrocarbon and peroxisome proliferator-activated receptors and oxidative stress response). While these streams were selected to cover a wide range of agricultural impacts, in addition to the expected pesticides, wastewater-derived chemicals and chemicals typical for street run-off were detected. The unexpectedly high estrogenic effects in many samples indicated impact by wastewater or overflow of combined sewer systems. The 128 water samples exhibited a high diversity of chemical and effect patterns, even for different rain events at the same site. The detected 290 chemicals explained only a small fraction (<8 %) of the measured effects. The experimental effects of designed mixtures of detected chemicals that were expected to dominate the mixture effects of detected chemicals were consistent with predictions for concentration addition by a factor of two for 94 % of the mixtures. Overall, the burden of chemicals and effects were much higher than previously detected in surface water during dry weather with the effects often exceeding effect-based trigger values.

Introduction

Surface waters can be impacted by a large number of organic micropollutants, including pesticides, pharmaceuticals and industrial compounds, which can enter the aquatic environment from both point sources, such as wastewater effluent discharge, and non-point sources, such as agricultural run-off. Small streams have large lotic biodiversity, but, in comparison to larger systems, can be disproportionally affected by chemical pollution due to smaller dilution ratios.[1] Pesticides from agricultural run-off reduced invertebrate biodiversity in streams in Australia and Europe[2, 3] and wastewater treatment plant (WWTP) effluents may also impact invertebrates.[4] Further, the ecological effects of pesticides on small streams generally increase after rainfall events due to run-off from agricultural areas.[5]

Several studies that have evaluated the risk posed by organic chemicals in small streams have focused on chemical analysis.[6, 7] Targeted chemical analysis is traditionally applied to monitor chemical water quality, but lacks information on effects of non-target chemicals or chemicals at concentrations below analytical detection limits. Still, these may contribute to the overall effect. In-vitro bioassays can be applied for water quality monitoring to detect the mixture effects of chemicals present in a sample. Combinations of in-vitro bioassays and chemical analysis have been applied mainly to larger water bodies,[8-11] with fewer studies addressing smaller streams and mainly under low flow conditions in dry weather.[12, 13] In contrast, during rainfall events, concentrations of pesticides and their transformation products have been observed to peak in small rivers.[14, 15] Given that substantial effects in in-vitro assays have been observed in collected stormwater,[16, 17] it is timely to ask the question how chemicals and their mixtures assessed by an in-vitro test battery fare during rain events in small streams.

We assessed the chemical burden in small agricultural streams during rainfall events using a battery of in-vitro bioassays to identify which mixture effects exceed acceptable levels and which types of chemicals are driving the observed mixture effects. Water extracts were collected from 44 sites throughout Germany, with multiple samples collected during different rain events at most sites. The studied bioassays covered different stages of cellular toxicity pathways, including induction of xenobiotic metabolism, hormone receptor-mediated effects and adaptive stress responses. Specifically, this included assays indicative of activation of the aryl hydrocarbon receptor (AhR), binding to the peroxisome proliferator-activated receptor gamma (PPARγ), activation of the estrogen receptor (ER) and oxidative stress response. These bioassays were responsive in surface water and wastewater,[10, 18, 19] with the endpoints also identified as most the responsive and therefore priority endpoints for surface water using the multiplexed Attagene assays that cover 69 endpoints.[18, 20, 21] The effect in the water extracts were compared with bioassay specific effect-based trigger values

(EBTs) derived from Environmental Quality Standards (EQS) from the European Union Water Framework Directive (WFD).[22] In addition to bioanalysis, chemical analysis of 395 chemicals including pesticides, pharmaceuticals and industrial chemicals was undertaken.

Iceberg modelling using the bioanalytical equivalent concentration (BEQ) approach was applied in the current study to determine the contribution of detected chemicals to the observed effect.[23] Bioanalytical equivalent concentrations from bioanalysis ($BEQ_{bio,iceberg}$) relates the effect of the sample to the effect induced by the assay reference compound, whereas bioanalytical equivalent concentrations from chemical analysis (BEQ_{chem}) are determined based on the concentration of a chemical in a sample and its relative effect potency (REP_i). BEQ_{chem} is similar to the toxic unit (TU) approach[24, 25] or exposure-activity ratio (EAR) approach,[21] and the different measures can be converted into each other.[26]

The BEQ concept is based on the assumption that the many chemicals in a mixture act in a concentration additive manner, which was appropriate to predict mixture toxicity in assays indicative of receptor-mediated effects, adaptive stress responses and cytotoxicity.[17, 27, 28] In the field, stress can exacerbate the mixture effects and lead to more-than additive effects,[29] but for large number of chemicals, as in our study, additive mixture models are considered as broadly applicable also in in-vivo assays.[30]

$BEQ_{bio,iceberg}$ and BEQ_{chem} can be compared to determine how much of the effect is explained by detected chemicals. In previous studies only a small fraction of the sample's effect in assays indicative of xenobiotic metabolism and adaptive stress responses could be explained by the quantified chemicals.[8, 10, 18, 31, 32] This is likely due to the thousands of non-quantified chemicals expected to be present in water samples[33] that may trigger these bioassays. To further explore which and how chemicals contribute to the known effect (i.e., the "tip of the iceberg"),[34] more than 200 synthetic mixtures of detected chemicals were run in the bioassays indicative of activation of AhR, binding to PPARγ and oxidative stress response. In contrast, for hormonal effects, a small number of potent hormone receptor agonists can typically explain the majority of effects,[35] and therefore no synthetic mixtures were measured in the assay for the activation of ER.

Materials and Methods

Sampling and sample processing. 128 water samples were collected from 44 sites in eleven German states from April to September 2018 (Table S1 of the Supporting Information) using a modified sampling device based on the technology introduced by Schulze et al.[36] Rain events causing water levels to rise by at least 5 cm in the streams triggered sampling. Two different sampling devices were used. One autosampler (Maxx Maxx Meß- und Probenahmetechnik GmbH, Rangendingen, Germany)

collected forty subsamples of 50 mL over a time period of 3 hours 20 minutes during the rain event with each subsample collected every 5 min (duration of sampling approximately 45 sec). The other sampling device was also triggered by rising water levels and collected up to 1 L of water in one bottle as described by Liess and van der Ohe.[37] The combined water samples of each rain event yielded a volume of up to 1 L or 2 L (less if the sampling device clogged), which was enriched after filtration using solid-phase extraction (SPE) with HR-X sorbent[38] with SPE process blanks run in parallel. For details on sampling sites, sampling and sample processing, see SI, Section S1.

Chemical analysis. 395 compounds (Table S2) were analyzed by liquid chromatography coupled to high resolution mass spectrometry (LC-HRMS) by direct injection as described in Section S2.

Bioanalysis. The extracts were run in four bioassays, AhR CALUX, PPARγ GeneBLAzer, ERα GeneBLAzer and AREc32 (see Table S4). All studied bioassays are mammalian reporter gene assays and were run in 384-well plates, with detailed methods provided in Neale et al.[32] and König et al.[10] In addition to the environmental extracts, individual chemicals found at high concentrations or expected to contribute to the effect were also run in the AhR CALUX (78 chemicals), PPARγ GeneBLAzer (43 chemicals) and AREc32 (87 chemicals) assays (all fingerprinted chemicals listed in Table S5). For all assays, cell viability in the mammalian cell lines was assessed in parallel to induction based on cell confluency using an IncuCyte S3 live cell imaging system (Essen BioScience, Ann Arbor, Michigan, USA).[19] Any concentrations that reduced cell viability by 10% or more (i.e., caused 10% or more cytotoxicity) were excluded from further data evaluation.

Data evaluation. Linear concentration-effect curves at effect levels up to 30% were used for data evaluation, with the concentration causing 10% effect (EC_{10}) derived for AhR CALUX, PPARγ GeneBLAzer and ERα GeneBLAzer and the concentration causing an induction ratio of 1.5 ($EC_{IR1.5}$) determined for AREc32. The concentration causing 10% inhibition (IC_{10}) was also evaluated using linear concentration-effect curves. Detailed information about the applied data evaluation approach is available in Escher et al.[39] The EC_{10} and $EC_{IR1.5}$ values were expressed as a relative enrichment factor (REF) in units of $L_{water}/L_{bioassay}$, while the EC_{10} and $EC_{IR1.5}$ values for the individual chemicals were given in molar units.

Iceberg modelling. Iceberg modelling using both the BEQ and TU approaches was applied in the current study to determine how much of the observed effect can be explained by quantified chemicals and how much is due to unknown chemicals (Figure 1). Sample EC values were converted to $BEQ_{bio,}$

$_{iceberg}$ using the EC value of the reference compound (Equation 1). BEQ_{chem} was calculated using Equation 2 by summing the BEQ_i of each quantified and bioanalytically characterized chemical. BEQ_i is the product of the concentration of the detected chemical (C_i) in molar units and its REP_i. REP_i was calculated using Equation 3 using the EC value of the detected chemical i and the EC value of the reference compound. Note that $BEQ_{bio,\ iceberg}$ was based on the effect of SPE extracts, whereas BEQ_{chem} was calculated from C_i using direct injection into the LC-HRMS, which is acceptable because generally good chemical recovery was observed previously for HR-X sorbent.[23] Hydrophilic compounds are likely to be poorly recovered by the HR-X sorbent, but these chemicals were not expected to contribute significantly to the observed mixture effect due to their typically much lower potency (Table S5). The EC values for the detected chemicals were either measured as part of this study or collected from the literature and the US EPA Tox21 database.[40] BEQ was expressed as benzo[a]pyrene equivalent concentrations (B[a]P-EQ) for AhR CALUX, rosiglitazone-EQ for PPARγ GeneBLAzer, 17β-estradiol equivalent concentrations (EEQ) for ERα GeneBLAzer and dichlorvos-EQ for AREc32.

$$BEQ_{bio,iceberg} = \frac{EC_y\ (ref)}{EC_y\ (sample)} \quad (1)$$

$$BEQ_{chem} = \sum_{i=1}^{n} BEQ_i = \sum_{i=1}^{n} REP_i \cdot C_i \quad (2)$$

$$REP_i = \frac{EC_y\ (ref)}{EC_y\ (i)} \quad (3)$$

The sample IC_{10} values were converted to $TU_{cytotoxicity(bio,\ iceberg)}$ using Equation 4 based on Müller et al.[13] TU based on chemical analysis ($TU_{cytotoxicity(chem)}$) was calculated using the detected chemical concentration and the IC_{10} value of the detected chemical i (Equation 5). IC_{10} values for analyzed chemicals were measured in the current study or collected from the US EPA Tox21 database (Escher et al. submitted). While not commonly applied for in-vitro bioassays, TUs from chemical analysis are often calculated for whole organisms, such as algae, daphnia and fish.[25]

$$TU_{cytotoxicity(bio,\ iceberg)} = \frac{1}{IC_{10}\ (sample)} \quad (4)$$

$$TU_{cytotoxicity(chem)} = \sum_{i=1}^{n} \frac{C_i}{IC_{10\ (i)}} \quad (5)$$

The percent contribution of individual detected chemicals i to the known fraction of effect (e.g., BEQ_{chem} or $TU_{cytotoxicity(chem)}$) was calculated using Equations 6 and 7.

$$\%\ \text{contribution of i to } BEQ_{known} = \frac{REP_i \cdot C_i}{BEQ_{chem}} \cdot 100\% \quad (6)$$

$$\%\ \text{contribution of i to } TU_{known} = \left(\frac{C_i}{IC_{10(i)}} \cdot \frac{1}{TU_{cytotoxicity(chem)}}\right) \cdot 100\% \quad (7)$$

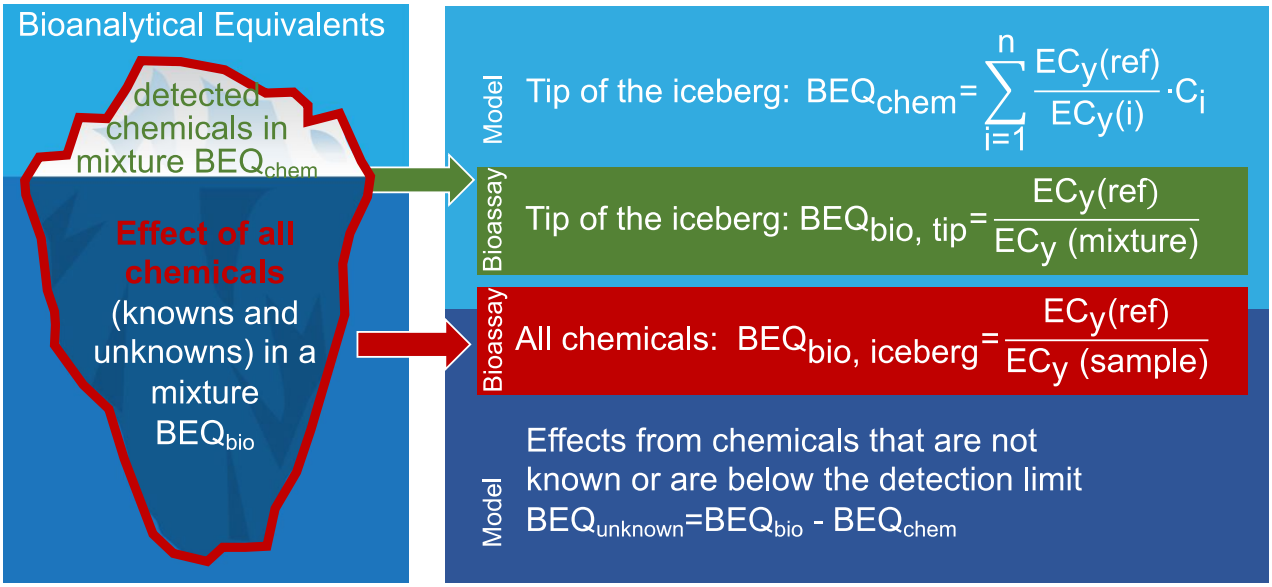

Figure 1: Bioanalytical equivalent concentrations from chemical analysis (BEQ_{chem}) are compared to the bioanalytical equivalent concentrations from bioanalysis ($BEQ_{bio,\ iceberg}$) using iceberg modelling. The contribution of detected chemicals to BEQ_{chem} (e.g., "tip of the iceberg") is determined both by modelling and using designed mixture experiments ($BEQ_{bio,\ tip}$). Y stands for the effect measure, e.g., y=10 for 10%, EC_{10}, or IR1.5 for $EC_{IR1.5}$.

Tip of the iceberg mixtures. Chemicals that dominated BEQ_{chem} were mixed in the ratios of concentrations they were detected in the samples. For activation of AhR 17 chemicals (1H-benzotriazole, 2-benzothiazolesulfonic acid, 2-hydroxybenzothiazole, 2,6-dichlorbenzamide, 5-methyl-1H-benzotriazole, 7-diethylamino-4-methylcoumarin, chlorotoluron, diflufenican, diuron, epoxiconazole, genistein, iminostilbene, isoproturon, MCPA, metamitron, pindolol, propylparaben) were mixed in 107 combinations of detected concentrations. Pindolol and 2,6-dichlorbenzamide were added because they had shown a positive response in the Tox21 database but our experiments showed no activity. Logistic reasons prohibited preparing matching mixtures for all water samples, but 107 of 128 mixtures were prepared. For PPARγ, we mixed 17 other chemicals (2-benzothiazolesulfonic acid, 2-hydroxybenzothiazole, 2,4-dichlorophenoxyacetic acid, 3,5,6-trichloro-2-pyridinol, 7-diethylamino-4-methylcoumarin, bezafibrate, chloridazon, desethylterbutylazine, diclofenac, losartan, MCPA, naproxen, prosulfocarb, prothioconazole-desthio, quinoxyfen, thiacloprid amide, triphenylphosphate) in 76 mixtures ratios as they were detected and one chemical (prothioconazole-desthio) turned out to be inactive during mixture experiments. For AREc32, 16 chemicals (2-benzothiazolesulfonic acid, 2-hydroxybenzothiazole, 2,4-dinitrophenol, 7-diethylamino-4-methylcoumarin, benalaxyl, desphenyl-chloridazon, dimethenamid, ethofumesate, flufenacet, genistein, iminostilbene, metazachlor, metolachlor, pethoxamid, propylparaben, triphenylphosphine oxide), one of which (benalaxyl) turned out to be inactive, were mixed in 44 mixture ratios. In addition, an equipotent mixture was prepared for all assays.

The stock solutions of the mixtures were prepared in DMSO from DMSO stocks of single compounds using a Tecan D300e Digital Dispenser (Tecan, Crailsheim, Germany). The effect concentrations of the mixtures EC_y(mixture) were reported in total molar concentration (of all 17 or 16 chemicals including the inactive ones) and converted to simulated REF by dividing by the total molar concentrations of these compounds in the water samples to yield EC_y(mixture) in units of REF. The $BEQ_{bio,tip}$ of the designed mixtures (Equation 8) were then compared with BEQ_{chem} and $BEQ_{bio,iceberg}$.

$$BEQ_{bio,\ tip} = \frac{EC_y(ref)}{EC_y(mixture)}$$

(8)

The index on prediction quality (IPQ, Equations 9 and 10) serves as a measure of how well experimental ($BEQ_{bio,\,tip}$) and predicted mixture effect ($BEQ_{chem,\,tip}$) agree, with an IPQ of 0 indicating optimal agreement.[27, 41]

$$\text{For } BEQ_{bio,tip} > BEQ_{chem,tip}\text{: } IPQ = \frac{BEQ_{chem,tip}}{BEQ_{bio,tip}} - 1 \quad (9)$$

$$\text{For } BEQ_{chem,tip} > BEQ_{bio,tip}\text{: } IPQ = 1 - \frac{BEQ_{chem,tip}}{BEQ_{bio,tip}} \quad (10)$$

Results and Discussion

Chemical analysis. 290 of the analyzed 395 chemicals were detected in at least one water sample (Table S2), with 10 to 144 chemicals detected per site. The industrial compound 2-benzothiazolesulfonic acid was most frequently detected and was found in 124 of the 128 samples (97% detection frequency). It is used in the production of rubber, is also a transformation product of mercaptobenzothiazole and its derivatives and has been previously detected in wastewater and surface water.[10, 42] It was also one of the most commonly detected chemicals in the Danube River.[31] In street run-off the concentrations of 2-benzothiazolesulfonic acid were up to 50 µg/L and thus 10 times higher than in wastewater or surface water, where it was present in similar concentration ranges as in the current study.[43] The chemical found at the highest concentration, with up to 126.2 µg/L (average concentration 11.2 µg/L), was oxypurinol, which is the pharmaceutical metabolite of the anti-gout pharmaceutical allopurinol, and has previously been found at concentrations up to 22.6 µg/L in German surface water.[44] The chemical profile also varied between sites and over time, with some sites dominated by pesticides and others containing higher concentrations of pharmaceuticals and personal care products (PPCPs) (Figure 2, Figure S1). A thorough evaluation of the chemical analysis is beyond the scope of the present study, which focuses on bioassays.

Bioanalysis. The observed effect in the activation of AhR, binding to PPARγ, activation of ER, oxidative stress response and cytotoxicity varied both between sites and within the same site over time (Figure 2 and Figure S2, see Table S6 for all EC values). For example, estrogenic activity varied

by almost a factor of one hundred in Site 22 between different rain events (Figure 2). Activation of ER was often the most responsive endpoint, followed by the responses of assays indicative of xenobiotic metabolism, activation of AhR and binding to PPARγ. The oxidative stress response assay was in many sites the least responsive.

While the studied small streams were in agricultural areas, five of the 44 sites (5, 26, 29, 35, 37) were directly impacted by municipal WWTP effluents and three others (sites 21, 22, 23) by industrial WWTPs (Table S1). Several other sites showed typical markers of wastewater, including the pharmaceutical carbamazepine and artificial sweeteners sucralose and saccharin (Table S1). A subset of these sites often had EC_{10} values less than one (i.e., effect observed after dilution) in the activation of ER assay pointing towards wastewater discharge (e.g., sites 13, 31 and 36). This suggests that water from water retention basins or combined sewer systems, where capacities were exceeded during rainfall events, entered streams or diffuse effluents from small upstream urban areas (Table S1) contributed to the effects.

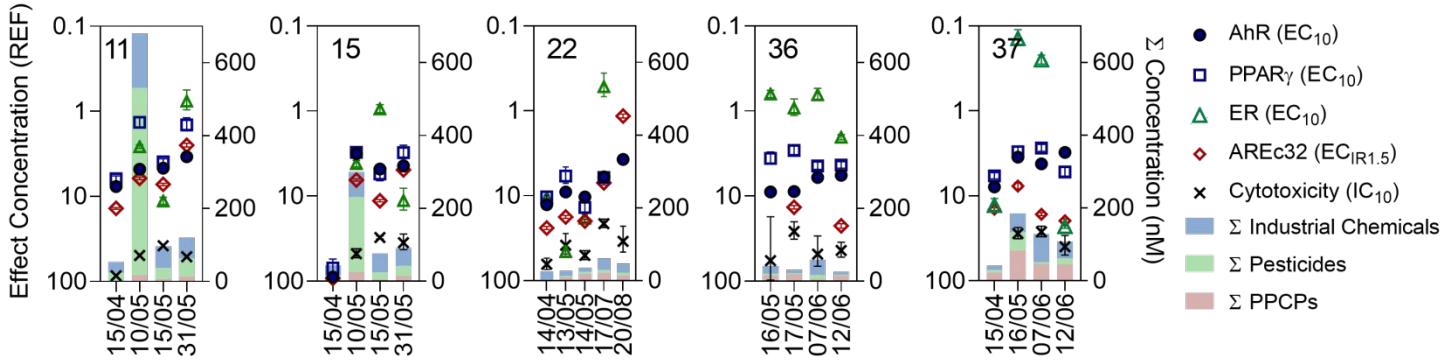

Figure 2: EC values for activation of AhR, binding to PPARγ, activation of ER and oxidative stress response (AREc32) for selected sites (11, 15, 22, 36, 37), with sum concentration of industrial compounds, pesticides and pharmaceuticals and personal care products (PPCPs) (nM). Cytotoxicity IC_{10} values are for the AhR CALUX, with IC_{10} values for the other assays provided in Table S6.

The level of activation of AhR and binding to PPARγ was similar to that previously observed in the German Ammer River, with EC_{10} REF values ranging between 2.0 to 35 and 1.1 to 90, respectively.[13] In contrast, estrogenic activity in the small streams was often higher than the observed effect in the Ammer River,[13] with many of the samples showing activity similar to wastewater effluent.[10, 18] The oxidative stress response was in a similar range as detected previously in streams and rivers in Australia, Germany and Switzerland.[12, 13, 18]

Comparison of measured effects in the water samples with effect-based triggers (EBT). The surface water extract EC values were converted to $BEQ_{bio,\ iceberg}$ values in units of ng or µg of reference compound per liter and were compared with preliminary surface water EBTs derived from the EU Water Framework Directive.[22] The preliminary EBTs, which were derived by reading across from the current environmental quality standards in the Water Framework Directive and applying a mixture factor where necessary, were updated with the newly available single chemical effect data (Table S5, no update of EBT for ERα GeneBLAzer) using the template provided by Escher et al.[22]

The EEQ of 79% of samples (Table S6) exceeded EEQ-EBT of 0.34 ng_{E2}/L for ERα GeneBLAzer[22] (Figure 3A), which was an unexpectedly high percentage, given that the sampling sites were selected with a focus on agricultural impact. However, chemicals usually associated with treated or untreated wastewater were detected at several sites (Table S1), which is consistent with the high EEQs. Previously, the EBT-EEQ had been able to differentiate clearly between wastewater and surface water with surface water rarely exceeding the EBT-EEQ.[22] The elevated estrogenic activity could be related to lower retention times in the WWTP and thus lower treatment efficacy and diffuse input of urban stormwater contamination from combined sewer systems. Rain events can also lead to dilution but since we only sampled during rain events, not the periods before and after the event, we cannot judge if dilutions by rain occurred. For example, sites 5, 21, 26, 29 and 35 were impacted by wastewater (Table S1) and all exceeded the activation of EBT-EEQ. In contrast, sites 22 and 37 also had WWTPs upstream of the respective sampling sites, but only exceeded the EBT during some rainfall events.

The EBT-B[a]P-EQ for AhR CALUX was published as 6.4 $ng_{B[a]P}/L$[14] but this value was only based on four experimental EC_{10} values. Using nine additional EC_{10} values (Table S5) brought the EBT-B[a]P-EQ to 4.3 $ng_{B[a]P}/L$, indicating the robustness of the initial derivation. The EC_{10} in Table S6 were converted to B[a]P-EQ with Equation 1 using the EC_{10} for B[a]P of 212 $ng_{B[a]P}/L$. 98% of the samples' B[a]P-EQ exceeded this EBT-B[a]P-EQ (Figure 3B). Prior experience with AhR CALUX in water samples is limited, but WWTP effluents[19] and wastewater-impacted rivers[13] had similarly high B[a]P-EQ values as many of the present water samples, while small streams unimpacted by wastewater had lower B[a]P-EQ levels.[13]

The EBT-rosiglitazone-EQ for PPARγ GeneBLAzer was previously 36 $ng_{rosiglitazone}/L$,[22] but was only based on data for three chemicals. With now six active chemicals the revised EBT-rosiglitazone-EQ amounted to 19 $ng_{rosiglitazone}/L$. Only 13% of the samples (Table S6) were compliant, with the remainder exceeding this EBT (Figure 3C). This is in contrast to a previous study, where only untreated wastewater exceeded the preliminary EBT for PPARγ, whereas surface water samples from the Danube River were compliant.[10] In another small stream, this revised EBT-rosiglitazone-EQ was

able to clearly differentiate between unimpacted stretches and tributaries of the river and WWTP effluent or thereby impacted stretches of the river.[13]

The EBT-dichlorvos-EQ for AREc32 remained virtually constant with 140 $ng_{dichlorvos}/L$ despite the database increasing from 11 to 21 chemicals. 60% of the samples exceeded this EBT-dichlorvos-EQ (Table S6, Figure 3D). Again, this EBT had previously differentiated well between more polluted water (wastewater and urban stormwater) and river water[22] and in another small stream study during dry weather, all sites, including those impacted by WWTP effluent were below the EBT-dichlorvos-EQ.[13]

This comparison with EBT-BEQs as well as with previous samples from wastewater and surface water suggests that many of the sites have a high chemical mixture burden, particularly concerning chemicals that activate AhR and ER.

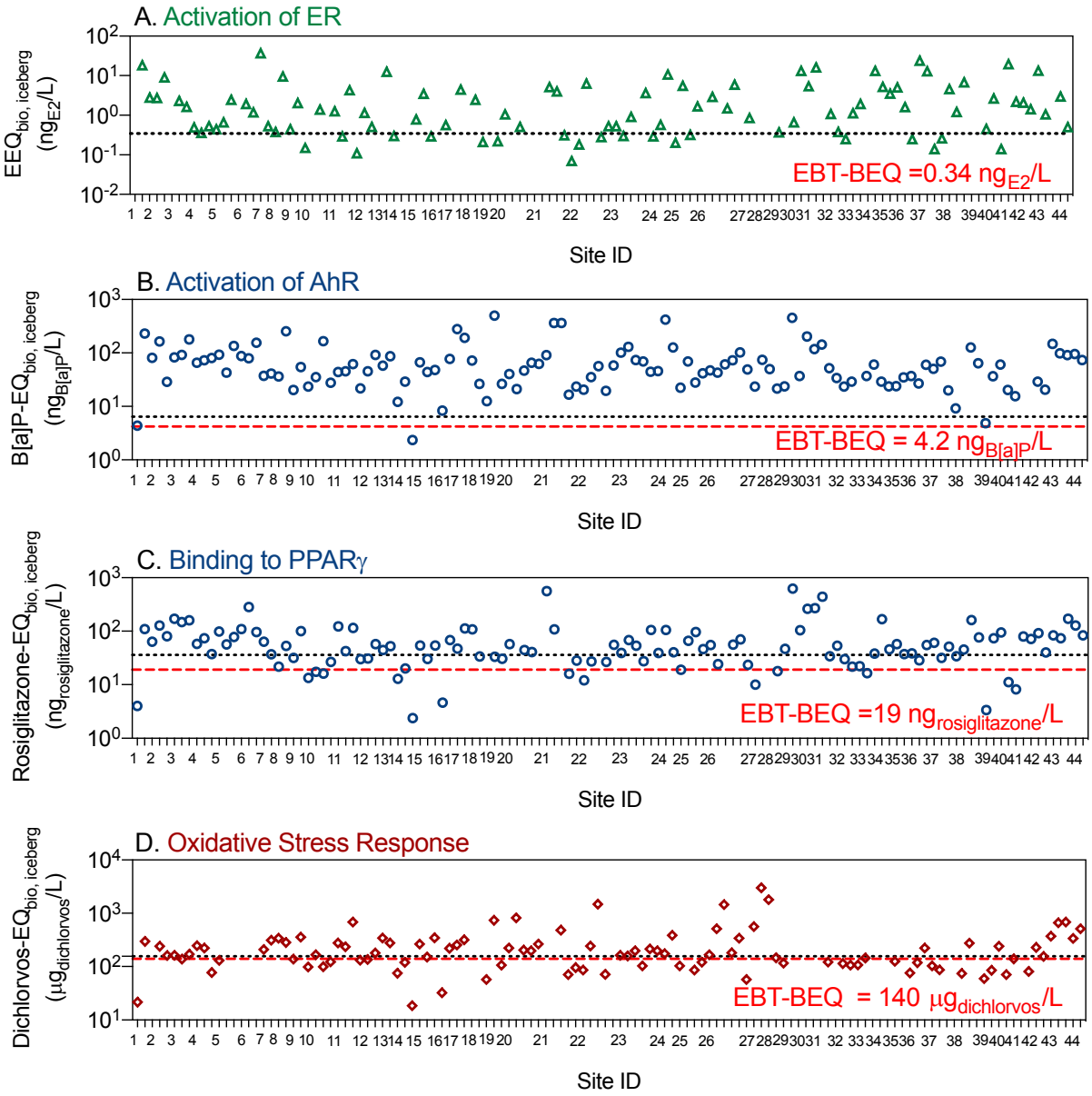

Figure 3: Comparison of water extract $BEQ_{bio,\ iceberg}$ values (ordered by site ID (Table S1)) with the preliminary effect-based trigger values (EBT) from Escher et al.[22] (dotted black lines) and the revised EBTs (red dashed lines).

Which chemicals are driving the effects in the water extracts? To better understand which chemicals are driving the observed effects, chemicals detected in the water extracts at high concentrations or expected to contribute to the effect in assays indicative of activation of AhR, binding to PPARγ and the oxidative stress response were fingerprinted. We omitted fingerprinting of single chemicals in the activation of ER assay because a small number of potent chemicals, namely natural and synthetic steroidal hormones, typically explain most of the effect in this endpoint.[45, 46] Bioanalysis is sufficient to characterize estrogenicity in water samples as the ratio of bioactive estrogens is typically fairly

constant in surface waters.[35] A wider range of chemicals are active in assays indicative of induction of xenobiotic metabolism and adaptive stress responses.[47] The IC_{10} and EC values for all chemicals measured in the current study or taken from literature are provided in Table S5.

For activation of AhR, effect measurements were available for 316 of the 395 analyzed chemicals (80%) using both experimental data and the Tox21 database. Of the 290 detected chemicals, effect data was available for 236 chemicals (81%), but most were not active (Table S5, Figure S3). EC_{10} values were available for 40 chemicals detected in the water extracts for the activation of the AhR assay. Nineteen of these values were from the Tox21 database, which used a different activation of AhR assay (rat cell line in the current study versus human cell line in Tox21 database). However, EC_{10} values for common chemicals run in both assays were generally within one order of magnitude (Figure S4), so both datasets were used to determine the effect based on chemical analysis, BEQ_{chem} (Table S7).

On average, 2-benzothiazolesulfonic acid explained 29.2% of the B[a]P-EQ_{chem} in the water extracts (between 0 to 98.2% explained), followed by the herbicide diuron (average 14.9%) (Figure 4A). The average contribution to B[a]P-EQ_{chem} is presented in Figure 4, but the contribution of each chemical to B[a]P-EQ_{chem} varied greatly for the different water extracts because the presence and concentrations of the individual chemicals varied considerably (Table S2) resulting in a wide range of B[a]P-EQ_i (Figure S5). For example, the industrial compound 7-diethylamino-4-methylcoumarin explained on average 4.8% of B[a]P-EQ_{chem} but contributed to over 95% of B[a]P-EQ_{chem} in all water extracts from the wastewater-impacted Site 37. 2-Benzothiazolesulfonic acid was one of the least potent chemicals in AhR CALUX (REP_i 5.67×10^{-6}), but it was present in all but two of the water extracts and was found at high concentrations (up to 6.4 µg/L). Therefore, not only highly potent chemicals but also chemicals present at high concentrations will contribute to the effect.

When comparing B[a]P-EQ_{chem} to B[a]P-$EQ_{bio,iceberg}$, only between 0.0004 to 2.79% of the effect could be explained by detected chemicals (Table S7). Previous studies have found between 0.2 to 71% of activation of AhR that could be explained by the quantified chemicals in surface water.[12, 31] These studies only had EC values for three to four of the detected chemicals, compared to 40 detected bioactive chemicals in the current study. AhR is mainly activated by hydrophobic organics such as polycyclic aromatic hydrocarbons. These bind to suspended particulate matter and would not be expected in the water sample filtered with a 0.7 µm filter but residual smaller particles and colloids may pass and be enriched by SPE, contributing to the unknown fraction of B[a]P-$EQ_{bio,iceberg}$. For these particles, a source in addition to road run-off, agricultural run-off and WWTP effluent will also be atmospheric deposition.[48]

Effect measurements were available for 310 out of the 395 analyzed chemicals for PPARγ GeneBLAzer, with data available for 232 of the detected chemicals (80%) (Table S5). However, only 9% of the detected chemicals tested in PPARγ GeneBLAzer were active, with REP_i values available for 20 chemicals (Figure S3). Diclofenac explained on average around a third (35.4%) of rosiglitazone-$EQ_{chem,}$ followed by 2-benzothiazolesulfonic acid (average 25.3%) and the herbicide MCPA (average 12.4%) (Figure 4B, Figure S6). Diclofenac was among the most potent chemicals measured in the PPARγ GeneBLAzer assay in the current study (REP_i 5.42×10^{-4}) and was also found at high concentrations (up to 1.3 µg/L). However, rosiglitazone-EQ_{chem} could only explain up to 1.66% of rosiglitazone-$EQ_{bio,iceberg}$ (average 0.18%) (Table S8). Detected chemicals have previously shown to explain a low fraction of the effect (<1%) in the PPARγ GeneBLAzer assay in surface water and wastewater[10] and spiked surface water.[23]

Bioassay data were available for either the AREc32 or ARE GeneBLAzer assays for 309 of the 395 chemicals analyzed. If both were available, only AREc32 was reported. Of the 290 detected chemicals, effect data was available for 233 chemicals (80%), with 52 of the detected chemicals active in the AREc32 (29 chemicals) or ARE GeneBLAzer assays (23 chemicals) (Table S5, Figure S3). The ARE GeneBLAzer data were collected from the US EPA Tox21 database and was expressed as an EC_{10} rather than an $EC_{IR1.5}$. The $EC_{IR1.5}$ and EC_{10} values for common chemicals were generally within an order of magnitude (Figure S7), but the REP_i values for chemicals run in ARE GeneBLAzer were calculated using the dichlorvos EC_{10} value from the Tox21 database.

2-Benzothiazolesulfonic acid explained 35.4% of dichlorvos-EQ_{chem} on average, followed by industrial compound 2,4-dinitrophenol (average 12.0%) and herbicide metolachlor (average 7.2%) (Figure 4C, Figure S8). Metolachlor was previously found to contribute to dichlorvos-EQ_{chem} for the oxidative stress response in wastewater effluent and surface water downstream of a WWTP in Switzerland.[12] On average, only 0.28% of dichlorvos-$EQ_{bio,iceberg}$ could be explained by dichlorvos-EQ_{chem} (Table S9). This is similar to previously observed for surface water and wastewater.[12, 27, 31] In one sample, 8b, 8% of dichlorvos-$EQ_{bio,iceberg}$ was explained by the potent herbicide pethoxamid (REP_i 2.66), which was detected at 13.1 µg/L.

While many different chemicals contributed to the BEQ_{chem} in the three assays, 2-benzothiazolesulfonic acid explained between 25.3 to 35.4% of BEQ_{chem} on average in the three assays. While 2-benzothiazolesulfonic acid was not particularly potent in any of the assays, the widespread presence and high concentrations (average concentration 1.1 µg/L) meant it was a dominant contributor to BEQ_{chem}. This suggests that future water quality monitoring studies should include 2-benzothiazolesulfonic acid, especially as it is also a marker of street run-off and as such

complements the traditional wastewater markers such as estrogenic hormones or pesticides as markers for agricultural inputs.

Other studies have also used in-vitro or in-vivo data to prioritize chemicals of concern. Focusing on assays included in the US EPA Tox21 database, Corsi et al.[21] found that the industrial compounds 4-nonylphenol and bisphenol A and the herbicides metolachlor and atrazine were among the chemicals identified as of greatest concern in water samples collected from the Great Lakes tributary. Metolachlor was also identified as a contributor to dichlorvos-EQ_{chem} for oxidative stress response in the current study. Further, many of the chemicals contributing to BEQ_{chem}, including the pharmaceuticals bezafibrate and diclofenac and the herbicides prosulfocarb and metolachlor, also ranked highly in a list of 214 chemicals present in European surface waters that potentially pose an acute hazard to fish, algae or crustaceans.[49]

Iceberg modeling of cytotoxicity is described and discussed in the SI, Section S5. Overall, a substantially higher fraction of cytotoxicity than of activation of specific effects could be explained because a larger number, i.e., 102, detected chemicals had experimental cytotoxicity IC_{10}: 0.2 to 122% for AhR CALUX, 0.2 to 22% for PPARγ GeneBLAzer and 0.02 to 8.8 % for AREc32 (Figure S10).

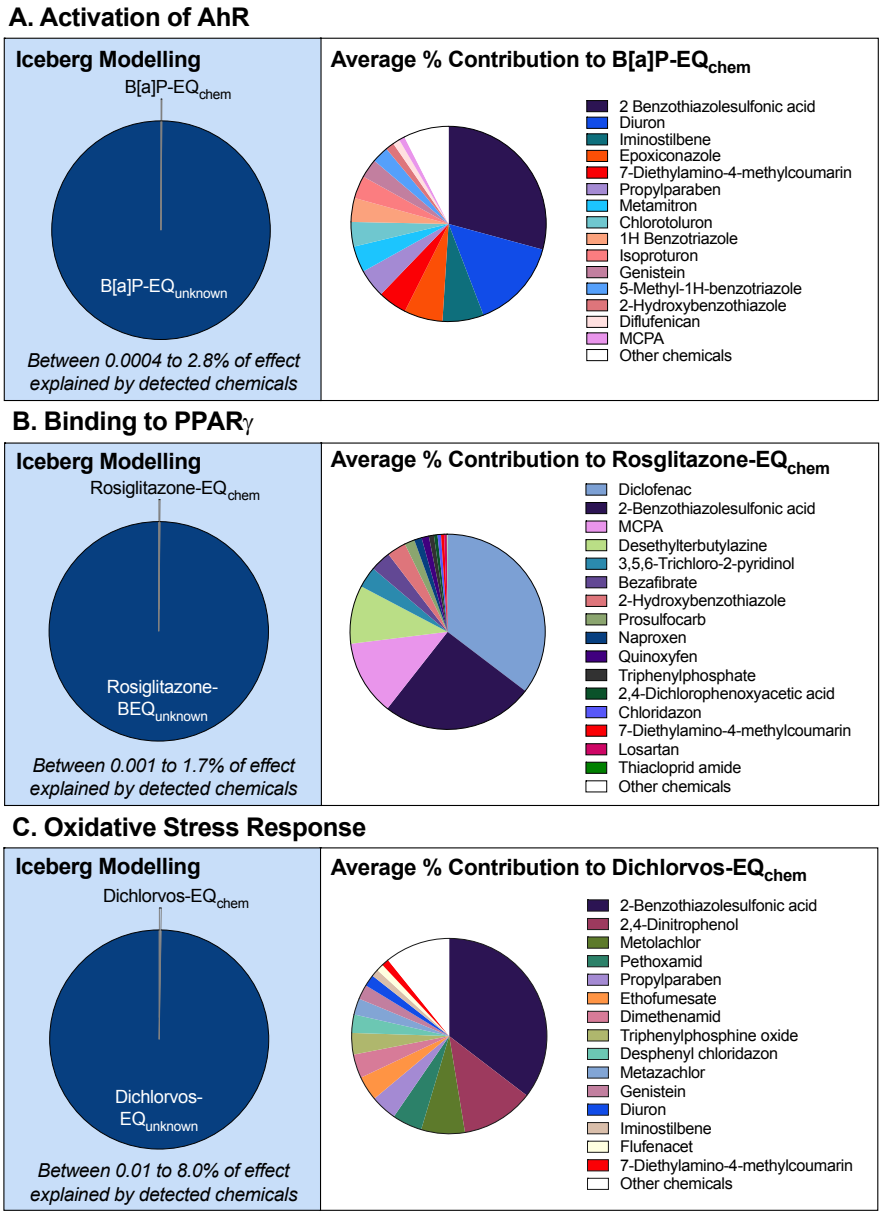

Figure 4: Average fraction of BEQ_{chem} that explained $BEQ_{bio,iceberg}$ (left) and top 15 to 16 chemicals contributing on average to BEQ_{chem} (right) for assays indicative of activation of (A) AhR, (B) binding to PPARγ and (C) oxidative stress response.

Equipotent mixtures of the detected chemicals. The concentration-response curve for activation of AhR of the equipotent mixture of the 15 chemicals that contributed most to the BEQ_{chem} agreed well with the prediction for concentration addition (Figure S11A) with an index of prediction quality (IPQ) of -0.11. This means that the chemicals detected are acting according to the mixture concept of concentration addition in mixtures. The equipotent mixture of PPARγ GeneBLAzer (Figure S11B) was much more potent than predicted for concentration addition with an IPQ of 3.69. This is especially surprising because the mixtures with the concentration ratios as detected in the water

samples were generally much closer to IPQ 0. The equipotent mixture of AREc32 (Figure S11C) had an IPQ of 0.46, which means that the experimental effect was higher than the predicted mixture effect. Various 5- to 10-component equipotent mixtures run in the AREc32 assay had IPQs around 0 confirming concentration addition but some mixtures had IPQ up to 1 indicating some variability.[27]

Tip of the iceberg mixtures. Since B[a]P-EQ_{chem} explained only a very small fraction of the B[a]P-EQ_{bio} (Figure S12A), it was checked by designed mixture experiments of chemicals in the detected concentration ratios of water samples if the detected chemicals act together according to concentration addition. The 107 reconstituted mixtures in AhR contained between 3 and 14 components in the detected concentration ratios. The selected 17 chemicals explained on average 93% of the overall BEQ_{chem} (min 26 %, max 99.9%). The concentration-response curves for activation of AhR are depicted in Figure S13 together with the predictions for CA. The EC_{10} values were converted to $BEQ_{bio,tip}$ and compared with $BEQ_{chem,tip}$ (Table S7, Figure 5A and S12B). With few exceptions, the agreement was within a factor of two, which is also reflected by the IPQ values (Table S7), which had a mean of 0.24 (95% CI 0.14 to 0.33, Figure S12C), indicating a slightly higher effect of the experimental mixture $BEQ_{bio,tip}$ than of the predicted $BEQ_{chem,tip}$. This small systematic deviation may be caused by the two chemicals that were inactive in the mixture experiments, whereas they had been reported active in Tox21. They may have been below their threshold of effect alone but contributed to the mixture effect.

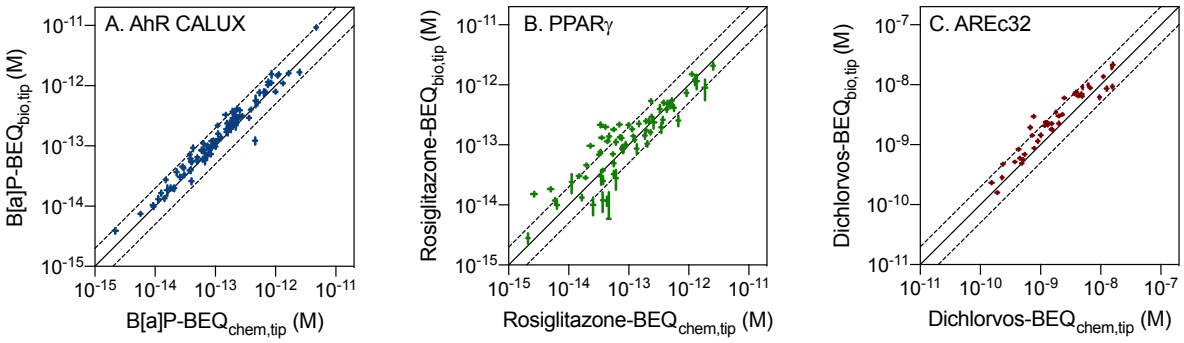

Figure 5: Agreement between $BEQ_{bio,tip}$ and $BEQ_{chem,tip}$ for (A) activation of AhR, (B) binding to PPARγ and (C) oxidative stress response. No symbols are shown, the lines at the points are the error bars (standard error), the full line is the 1:1 relationship and the dashed lines indicate 2:1 and 1:2 ratios.

The 76 mixtures of the 17 chemicals with the highest predicted rosiglitazone-EQ_{chem} in concentration ratios of the water samples (Table S8, concentration-response curves (CRCs) in Figure S14) yielded IPQs ranging from -6.9 to 5.4, with a mean of 0.32 but the 95% CI only ranged from -0.04 to 0.70,

which indicates that the majority of IPQs is above 0, indicating more potent mixtures than expected (Figure S15C). The relationship between rosiglitazone-$EQ_{chem,tip}$ and rosiglitazone-$EQ_{bio,tip}$ showed more variability than in AhR CALUX but the values are within a factor of two around the one-to-one line (Figure 5B). The higher variability between prediction and measurement is caused by the generally higher variability of individual data points in the CRCs of this assays, which is due to a larger background signal and hence lower signal-to-noise ratio.

The deviation from the relationship between dichlorvos-$EQ_{bio,tip}$ and dichlorvos-$EQ_{chem,tip}$ was well within a factor of two (CRCs in Figure S16, Figure 5C, Table S9) but directed towards higher experimental effects similar to AhR. Hence the deviation towards higher potency experimentally as compared to the mixture model of concentration addition appears to be small but consistent and might be caused by some imprecision of the single chemicals' EC_{10} values or the one inactive chemical benalaxyl. The IPQ values of the 44 mixtures (Table S9) ranged from -0.69 to 3.5 with a mean of 0.51 (95% CI 0.30 to 0.59, Figure S17C).

In summary, over all the 227 mixtures the mixture components appeared to act fairly close to concentration-additive in all three in-vitro bioassays, confirming that the BEQ concept is applicable to these bioassays and types of samples. The IPQs were close to 0 with a tendency to positive values for AhR CALUX (Figure S12C) and AREc32 (Figure S17C), even more for PPARγ GeneBLAzer (Figure S15C), which points to experimental effects being slightly higher than predicted, but the IPQ values did not shown any correlation to the composition of any of the mixtures.

Outlook. It has been demonstrated previously that a complete pesticide screening is required to estimate the surface water quality of small streams[50] and, while individual pesticides might exceed chemical-specific water quality criteria, it is really the mixture effect that needs to be considered to understand ecological effects[51] and risk.[7] Pesticides drive the risk predicted with the method of multi substance potentially affected fraction (msPAF) even in wastewater impacted streams at low-flow conditions.[52]

But the situation might change dramatically during rain events as described here, where we recorded a high spatial and temporal variability. While further studies on exceedance of chemical-specific water quality criteria and the ecological impact and in-vivo toxicity of the described rain events are forthcoming, the focus on present study was on the in-vitro assays and biological endpoints most commonly impacted by water-borne pollutants.

We demonstrated that non-pesticide chemicals and even typical wastewater-derived chemicals were found at sites assumed prior to the study to be largely free from wastewater effects. All observed in-vitro effects were dominated by street run-off chemicals such as 2-benzothiazolesulfonic acid.

Previous effect studies on stormwater demonstrated that effect levels were similarly high as WWTP effluent and all urban stormwater samples investigated showed estrogenic effects.[17] Rain events clearly pose a threat to water quality in small streams and analysis of pesticides alone cannot adequately judge the toxicological impact unless analytical monitoring is complemented by bioassays.

ASSOCIATED CONTENT

Supporting information

The supporting information is available free of charge at https://pubs.acs.org/doi....

Additional information on chemical analysis, bioassays, iceberg modelling of effects and cytotoxicity, equipotent mixture experiments, designed mixture experiments (pdf). Excel file with all experimental data.

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Author Contributions

MaL lead the sampling study; WB and RBS contributed conceptually and to site selection and sampling, TS, ML developed the sampling device; TS designed and programmed the target screening software, MaL, MoL, LL, RBS, VS, PV and OW contributed to monitoring coordination, site selection, sampling and evaluation of wastewater influence; EC and MKr performed the chemical analysis and data evaluation; RG, MoL and VS, extracted all samples; MKö and RS performed the bioassay experiments; GB performed and evaluated the tip of the iceberg mixture experiments; BE conceived the bioassay study, developed all data evaluations and models; PN evaluated all bioassay data, performed the iceberg modeling; PN and BE wrote the manuscript; all authors reviewed the manuscript.

All authors have given approval to the final version of the article.

Notes

The authors declare no competing financial interest.

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